# SEROLOGICAL STUDIES OF EMBRYOGENESIS IN ECHINOIDS

By

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# I. INTRODUCTION

The manner in which cells of apparently identical genotype become morphologically and functionally divergent is one of the central problems of developmental biology. The relative roles of the nucleus and cytoplasm in cell differentiation have been the subject of much fruitful speculation and investigation. Since 1941, when Beadle and Tatum, working with Neurospora, demonstrated that a single gene is responsible for production of a single enzyme, the investigations of many embryologists have been focused on enzymes, and on proteins in general, as the functional and structural expressions of differential gene activity in the developing embryo. With the demonstration by a number of workers that DNA represents the genetic material, and the subsequent explosion of ideas concerning its structure, replication (Watson and Crick, 1953), transcription into RNA (Brenner, 1961), and translation into protein (Nirenberg and Matthaei, 1961), the mechanisms by which DNA directs the amino acid sequence of proteins have been clarified. Increased knowledge of protein secondary structure (Pauling and Corey, 1951), and the evidence that determination of primary structure regulates the secondary and tertiary structure of a protein, and thus some of its characteristics and function (Helinski and Yanofsky, 1966), have further encouraged the study of proteins as direct expressions of gene activity in development. The theories developed by Tyler (1947) and Weiss (1947) implicating the antigen-antibody-like interaction of complementary macromolecular substances with the causes of

growth and differentiation have acted as a stimulus to much of the modern work concerning the role of proteins in embryogenesis.

Much of the initial work on proteins in embryonic development focused on the level and time of appearance of enzyme activities (Gustafson and Hasselberg, 1951), or on the enzymatic changes occurring in the development of a particular organ (Moog, 1966). Such studies are limited by the enzyme assay techniques available. Furthermore, the appearance of enzyme activity may result from enzyme activation and may be far removed from gene expression and production of the protein itself. Moog (1966) points out that while as a rule developing tissues have distinctive and changing enzyme patterns peculiar to the tissue, it is difficult to associate these patterns with the causes of differentiation.

Because of their sensitivity, immunological methods have been extensively used in the study of qualitative and quantitative changes in proteins in a variety of developing embryonic systems. Using antisera to adult chicken serum, Schechtman (1947) demonstrated antigens[1] in chick embryos and yolk similar to those found in adult serum. Absorption with yolk removed the reactivity of the antisera to primitive streak embryos but not to the homologous antigen (adult chicken serum) or older (5-day) chick embryos. Proteins similar to those found in the adult were

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[1] The term antigen will be used throughout this dissertation, as it has been by the majority of authors cited here, with the full knowledge that what is being detected by the antisera is a specific combining or determinant group on the antigen molecule (Landsteiner, 1962). Identity between antigens must thus be interpreted with care, since two different substances may have some common determinant groups. Antigenic molecules from cell homogenates, capable of stimulating the production of precipitating antibody in rabbits are, in these cases, most probably proteins or protein complexes such as ribonucleoproteins, mucoproteins, or lipoproteins.

detected in embryonic serum by five to six days of incubation (Nace and Schechtman, 1948). These proteins, present in the yolk and early embryo, appear to be synthesized by the mother and transferred to the yolk of the embryo with little apparent alteration. The maternally synthesized proteins are probably the source of early serum proteins of the chick (Nace, 1953; Schechtman, 1956).

The use of adult organs as injection antigens, and absorption of the resulting antisera with heterologous organs has allowed the production of highly selective antisera. Ebert (1951) used organ-specific sera to study the appearance of adult antigens in the spleen and brain of 9-, 12-, and 18-day chick embryos. Both specific and shared antigens characteristic of the adult organs were found in the three stages studied. In addition, one spleen-specific antigen not found in spleen extracts from 9- and 12-day embryos appeared in the spleen extracts from the 18-day embryo. Mid-streak stage chick embryos are the youngest stage capable of giving a detectable reaction with antiserum specific for cardiac myosin (Ebert, 1953). Initially, extracts of all areas of the epiblast contained specific combining groups and thus appeared to be synthesizing myosin. However, by the head process stage, the reactive groups were confined to the heart-forming areas. Similar studies on the appearance of adult organ antigens have been done on chick liver (Croisile, 1960), duodenum (Van Alten, 1959), and erythrocytes (Briles, McGibbon, and Irwin, 1948). The general picture which emerges is one in which the embryonic organs acquire more of those antigens characteristic of the adult organ. Such studies, however, have not dealt with possible embryonic antigens which may be

involved in differentiation and disappear before adult status is reached.

The work of Weller and Schechtman (1962) has demonstrated electrophoretically at least two embryonic types of serum protein, an embryonic $\alpha_2$ globulin and an embryonic $\gamma$ globulin, in the chick. These embryonic types disappear before hatching as the pattern of serum proteins synthesized changes from one embryonic in character to that of an adult.

Hemoglobin synthesis has been studied in chick embryos. In addition to the adult type(s) which appears at 48 hours, and a fetal type which appears at about 28 hours and persists at a low level even in the adult, an immunologically distinct hemoglobin, present only during embryonic life, is detectable. It appears at 28 hours and disappears by 36 to 48 hours of incubation (Wilt, 1962).

Most of the studies of chick embryos discussed were concerned with the synthesis of proteins in embryos long after differentiation of cell types and organogenesis has begun. However, much of the amphibian work is concerned with the proteins and antigens present or synthesized in the embryo during early development before and during the process of differentiation. Cooper (1946, 1948, 1950) has successfully demonstrated the antigenicity of Rana pipiens embryos and has detected antigens related to but not identical with adult serum proteins in the eggs and embryos of this species. Flickinger and Nace (1952) have demonstrated one or more antigens in extracts of hatched tailbud (stage 20-21) embryos of R. temporaria, not detectable in the extracts of previous stages. They were, however, unable to identify these antigens electrophoretically. Antiserum against extracts of tailbud embryos produced inhibition of development, gradient-wise cytolysis, and death by the hatched tailbud stage,

in frog embryos cultured in dilutions of this serum. The embryos developed normally in dilutions of preinjection sera or antisera to earlier stages. The antibodies present in the antiserum against tailbud embryos, and apparently not present in antisera against previous stages, may be produced in response to a newly synthesized antigen(s) or in response to an antigen(s) which has changed solubility or location.

Spar (1953), working with R. pipiens embryos, found both qualitative gains and losses in the saline-soluble antigens detectable during the transition from the blastula to the neurula. Embryos of Triturus alpestris appear to synthesize antigenic material between the blastula and gastrula stages, before neurulation, and between neurulation and the formation of the tailbud (Clayton, 1953). This study also demonstrated antigens specific to ectoderm and mesoderm. These antigen types coexist in the blastula, in tissues whose prospective fate is either ectoderm or mesoderm, but appear only in their specific tissue in the gastrula.

Spiegel (1960) examined extracts from developmental stages and from four adult organs of R. pipiens, using paper electrophoresis. He detected no changes during development except for the loss of two cationic bands by the hatched tailbud stage. The embryonic patterns were more complex than those from adult intestine, brain, skeletal muscle, and blood. Many of the cationic bands did not appear in any of the extracts of adult tissues. One possible interpretation of these results is that differentiation may, in part, involve loss of ability to synthesize particular proteins, or losses in extractable antigens due to changes in antigen location or solubility.

Shore (1965) has analyzed the saline-extractable proteins of embryonic stages of R. pipiens, using polyacrylamide gel disk electrophoresis and found changes only in band intensity. The R. pipiens x R. sylvatica[1] hybrid, which ceased differentiation at the early gastrula stage and dies one to two days later, differed from R. pipiens in the staining intensity of cathodic bands, probably histones. In addition, the hybrid possessed an extra cathodic band. Since these changes appear after hybrid development is arrested, the author believes that they do not represent primary defects. No studies of the paternal parent were made.

Certain conclusions can be drawn from these studies. Antigens are found in the embryo which are similar to or identical to those found in the adult. These antigens may be present initially due to their synthesis by the mother and subsequent deposition in the yolk of the oocyte, or they may be synthesized by the oocyte itself. Other adult antigens are not found in early embryos but appear either due to synthesis or solubilization as differentiation and organogenesis proceeds. In certain investigations, organ-specific or tissue-specific antigens have been demonstrated in embryos before the actual appearance of the organ or tissue itself. Embryos may possess proteins or antigens not found, at least in the same form, in the adult. Differentiation may proceed accompanied not only by gains in antigens or proteins, but by their loss. The pattern of protein types found in embryo extracts is often more complex than that of adult

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[1] All hybrids referred to in this dissertation will be designated in the conventional manner, with the maternal species given first and the paternal species given second.

tissues. In spite of the many experimental analyses it has thus far been difficult to correlate causal relationships in differentiation with qualitative and quantitative changes in embryonic proteins.

A closer examination of the role of the zygote nucleus in the production of proteins necessary to the developing and differentiating embryo has been possible using echinoid embryos. Mature unfertilized sea urchin eggs synthesize proteins at a measurable, but much lower rate than that exhibited during oogenesis or after fertilization. This synthesis probably involves turnover processes concerned with cell maintenance (Tyler, Tyler, Piatigorsky, 1968). Upon fertilization, protein synthesis increases considerably (Hultin, 1950; Nakano and Monroy, 1958). It proceeds at a high rate through cleavage to the early blastula and after a plateau increases again beginning at the mesenchyme blastula stage (Giudice, Vittorelli, and Monroy, 1962). This protein synthesis is a necessary prerequisite to the development of the sea urchin embryo. Treatment of the fertilized sea urchin egg with puromycin, which inhibits the incorporation of amino acids into protein (Yarmolinsky and de la Haba, 1959), immediately prevents cleavage and subsequent development (Hultin, 1961a). The failure of cleavage has been attributed primarily to failure to synthesize proteins playing either a structural or catalytic role in the operation of the spindle (Hultin, 1961a; Gross and Cousineau, 1963). In addition, puromycin appears to inhibit synthesis of proteins necessary for incorporation of thymidine into DNA (Black, Baptist, and Piland, 1967).

Parthenogenetically activated sea urchin eggs, which are probably haploid (Tennent, 1912a) synthesize proteins (Giudice and Monroy, 1958).

They also develop into pluteus larvae, although generally in low percentages. Nonnucleate egg fragments, obtained by centrifugation and activated parthenogenetically, may cleave to form solid unhatched blastulae (Harvey, 1936). These activated nonnucleate fragments are initially capable of the incorporation of amino acids into protein to an extent similar to incorporation by activated nucleate fragments or by fertilized nonnucleate fragments (Brachet, Ficq, and Tencer, 1963; Denny and Taylor, 1964). The proteins produced are qualitatively and quantitatively comparable to those produced by fertilized whole eggs (Tyler, 1966). Thus, it appears that initial protein synthetic activity can occur in the absence of nuclear DNA, although cytoplasmic DNA is present in the nonnucleate halves (Piko, Tyler, and Vinograd, 1967).

Further evidence for the lack of DNA dependence of early protein synthesis has been obtained through experiments utilizing actinomycin D, which inhibits DNA-dependent RNA synthesis (Reich, et al., 1961). Treatment of fertilized sea urchin eggs with actinomycin D at levels which inhibit messenger RNA synthesis, fails to inhibit protein synthesis by the cleaving embryos until the late blastula stage. The actinomycin D-treated embryos do not develop beyond the early blastula stage, although cell division continues for several hours after obvious differentiation has ceased (Gross and Cousineau, 1964). Messenger RNA is synthesized in the period immediately following fertilization (Nemer, 1963) and is associated with ribonucleoprotein particles, lighter than ribosomes, and also with light polyribosomes. Neither of these particles is active in incorporating labeled amino acids into protein at this stage (Spirin

and Nemer, 1965). Protein synthesis takes place in association with heavy polyribosomes which are not associated with the newly synthesized messenger RNA. Thus, the messenger RNA synthesized immediately after fertilization is not of a species necessary to early development or responsible for the initial protein synthetic activity of the embryo (Spirin and Nemer, 1965), but is probably necessary to and active in the embryo after hatching.

The mechanism of activation of protein synthesis upon fertilization, as well as the apparent inhibition of protein synthesis in unfertilized sea urchin eggs has been intensively investigated by a number of laboratories. Cell-free amino acid incorporating systems from unfertilized eggs exhibit a low level of synthesis as compared with the incorporation by cell-free systems from fertilized eggs (Hultin and Bergstrand, 1960). Particulate and supernatant fractions of unfertilized eggs have been reciprocally exchanged in cell-free amino acid incorporating systems. Ribosomes from unfertilized eggs appear to be the limiting factor, since they remained inactive when given fertilized egg supernate (Hultin, 1961b). In contrast, the synthetic messenger RNA, polyuridylic acid, stimulated in vitro incorporation of labelled phenylalanine into protein to the same or higher levels using unfertilized egg ribosomes as compared to ribosomes from fertilized eggs (Nemer, 1962; Tyler, 1963). Monroy, Maggio, and Rinaldi (1965) pointed out that polyuridylic acid has a binding affinity for ribosomes that is much greater than that of natural messenger (Okamoto and Takanami, 1963). In further experiments on this point, Maggio, et al. (1964) have demonstrated

*in vitro* that RNA extracted from unfertilized eggs stimulates amino acid incorporation, using ribosomes from sea urchin embryos or rat liver. Neither RNA from unfertilized sea urchin eggs, from sea urchin embryos, or from rat liver had any stimulating effect on ribosomes of unfertilized eggs.

Whether or not ribosomes from unfertilized eggs need be considered in an inhibited state cannot at present be decided, in view of their differential response to natural or synthetic messengers. In any case there seems to be adequate evidence from the experiments on activated anucleate halves, from actinomycin D-treated fertilized eggs, and from the work of Maggio, et al. (1964) that unfertilized eggs contain a store of messenger RNA. It is probably present in some inactive form; it is capable, upon activation, of sustaining protein synthesis in the fertilized egg, and it is responsible for most or all of the protein synthesis taking place up through the blastula stage. Experiments by Monroy, et al. (1965) demonstrated the activation of endogenous incorporating ability in unfertilized egg ribosomes by trypsin treatment. They suggest that messenger RNA, synthesized during oogenesis, becomes attached to ribosomes in some inactivated state, and is activated upon fertilization, possibly by the action of proteases.

The types of proteins synthesized during sea urchin development have been investigated by a number of workers. Spiegel and Spiegel (1959) analyzed the extractable proteins from *Arbacia punctulata* by free boundary electrophoresis. They determined that the extractable proteins of blastulae, gastrulae, and plutei gave essentially identical electrophoretic

patterns. Monroy, Vittorelli, and Guarneri (1961) studied soluble proteins of Paracentrotus lividus, using starch electrophoresis and column chromatography and found no qualitative changes in development. Spiegel, Ozaki, and Tyler (1965) conducted studies of radioactively labeled proteins extracted from Strongylocentrotus purpuratus, using polyacrylamide gel disk electrophoresis; newly synthesized proteins in early cleavage or in prism embryos, grown with and without actinomycin D-treatment, were examined. The patterns from actinomycin D-treated embryos were qualitatively identical with controls, although in the prism embryos the quantity of proteins synthesized was greatly reduced. In addition, they found no significant qualitative differences between patterns of the two stages studied.

Terman and Gross (1965) performed essentially the same experiment on A. punctulata. Using a slightly different method of assay, they found qualitative shifts in the patterns of labeled proteins synthesized in actinomycin D-treated embryos paralleled that of controls up to the blastula stage. The pattern of proteins synthesized in the treated embryos began to shift after the blastula stage, and was no longer comparable to the control pattern. This substantiates an earlier report by Gross (1964) that untreated blastulae synthesize large quantities of a protein class synthesized only in small quantities by actinomycin D-pulsed blastulae.

Some more specific analyses for protein types have been performed. Pfohl and Monroy (1962) assayed for esterases following polyacrylamide gel electrophoresis. They demonstrated a new esterase band in the plutei of A. punctulata. They also found a new band of alkaline phosphatase

activity first detectable in mesenchyme blastulae. Silver and Comb (1967) studied acetic acid soluble proteins of Lytechinus variegatus and demonstrated an increased synthesis of one nuclear protein fraction, probably histone, at the beginning blastula stage.

In immunological studies of Paracentrotus lividus embryos, Perlmann and Gustafson (1948), using ring precipitation tests, and Perlmann (1953) using the Ouchterlony technique demonstrated antigen(s) in saline extracts of plutei which were not present in saline extracts of unfertilized eggs. These antigens were first detectable in the mesenchyme blastula stage. Aside from these qualitative differences, which may reflect differences in solubility, the spectrum of saline soluble antigens is more or less the same for the stages studied, with the major portion of the antigen population common to all stages.

Perlmann and his coworkers have conducted a series of studies on some of the soluble protein antigens present at all stages in P. lividus. They found two antigens to be associated with yolk granules. The amounts of these yolk antigens present in the embryos decrease as development proceeds. They also found one antigen to be associated with a microsomal fraction (Perlmann and Couffer-Kaltenbach, 1957; 1964).

In a more recent paper, Westin, Perlmann, and Perlmann (1967) have combined the techniques of $^{14}C$ labeling of proteins synthesized by P. lividus eggs and embryos with analysis of extracts of these embryos by immunodiffusion and immunoelectrophoretic techniques, followed by autoradiography of the resulting dried plates. They confirm the findings of other laboratories (Terman and Gross, 1965) that the types of proteins

synthesized shift as development progresses. They report synthesis by the embryos of antigens present only in the egg or in the pluteus and qualitatively different from those newly synthesized antigens common to all stages.

From these studies it appears that developing embryos synthesize several major classes of proteins during development. Although different quantities of these protein classes are selectively synthesized at different times in development, qualitative changes are not easily detectable, except with specific assay methods. Some antigens seem to be either newly synthesized or newly soluble in later developmental stages and some enzymes are either newly synthesized or newly activated. The question of whether these new molecular types are synthesized at the direction of the zygote nucleus, or by messenger RNA already in the cytoplasm, requires some additional marker of nuclear activity.

Hybrids have long been considered a useful tool in exploring the relationship of the zygote nucleus to the cytoplasm in development. In morphological studies of a large number of hybrid sea urchin and amphibian embryos, cleavage rate appears to be regulated by the maternal cytoplasm (Tennent, 1908; Moore, 1933; Moore, 1941). Influence of the paternal sea urchin nucleus was first observed at the mesenchyme blastula stage as in the cross between Cidaris tubuloides and L. variegatus. In this hybrid the primary mesenchyme is formed earlier than is normal to the maternal species. This and its site of formation are both paternal traits (Tennent, 1914). Reciprocal crosses between R. pipiens and R. palustrus produce embryos in which influence of the sperm is detectable as early as

neurulation. This is manifested by an intermediate rate of development, which in the R. palustrus x R. pipiens hybrid is faster than the maternal rate. The developmental period from late blastula to early gastrula is the period during which many types of hybrids either die or at least cease to differentiate (Baltzer, 1952). However, other hybrids live beyond this stage and successfully differentiate into larvae. Some hybrid pluteus larvae show definite paternal characteristics of shape and skeleton (Tennent, 1911).

Analyses of respiratory rate and DNA content were made on the hybrid between P. lividus and A. lixula, which ceases development during gastrulation, and dies after living in an arrested stage for approximately 24 hours. Respiratory rate and DNA content of the paternal parent are lower than in the maternal parent. The hybrid values coincide with those of the maternal parent until the early blastula stage after which they are retarded to a level approximately midway between the two parents. This level is maintained until cellular degeneration begins. The authors (Whiteley and Baltzer, 1958) discuss two possible interpretations of these findings. Either the hybrid values are an average of those directed by each parental genome or the reduced values are the result of developmental disharmony or competition. Their results support the former interpretation more completely.

Flickinger (1957) has studied alkaline phosphatase levels in the S. purpuratus x Dendraster excentricus hybrid, and reports that the levels found in the hybrid plutei are higher than those of the maternal parent. Since the paternal species has higher alkaline phosphatase activity, his

data may be interpreted to indicate that the paternal nucleus is acting to either synthesize more enzyme or enzyme of higher activity. Such data indicate zygote nuclear activity.

A single set of experiments has been performed, in which proteins produced in sea urchin hybrids were examined in an attempt to clarify the role of the cell nucleus in early embryonic development. Harding, Harding, and Perlmann (1954) have assayed developmental stages of three sea urchin hybrids for antigens characteristic of the paternal species. In one of the hybrids studied, a strong precipitin band, obtained by the Ouchterlony technique was evidence for the presence of a paternal antigen. The other two hybrids each showed a weak paternal band. These antigens were detected as early as the mesenchyme blastula stage.

Assuming that genomic control of differentiation in a developing embryo is mediated ultimately through cellular proteins, the role of the zygote nucleus in directing the production of embryonic proteins must be clarified. With the exception of the as yet unconfirmed demonstration of paternal antigens in sea urchin hybrid mesenchyme blastulae (Harding, et al. 1954) there has been no direct evidence that the zygote nucleus directs the synthesis of new proteins during development. Therefore, the present research was undertaken to investigate the pattern of proteins present in embryos of two sea urchin hybrids, the intergeneric hybrid, L. variegatus x Tripneustes esculentus and the interordinal hybrid, S. purpuratus x D. excentricus. Serological assays of these hybrids were performed to determine whether there were gains or losses of specific proteins during development, whether any paternal proteins were detectable,

and whether any new hybrid proteins were produced. The protein patterns present in the unfertilized eggs and embryonic stages of the four paternal species were assayed in order to provide the background necessary to identify the proteins of each species and to characterize the protein types present in normally developing, homologously fertilized embryos. Serological methods such as the Ouchterlony method, immunoelectrophoresis, and passive cutaneous anaphylaxis were used in connection with specific absorptions of the antisera, since these methods combine high sensitivity with the ability to detect a variety of specific antigens in a complex mixture.

## II. MATERIALS AND METHODS

The sea urchins Lytechinus variegatus[1] and Tripneustes esculentus were collected on Virginia Key, Miami, Florida, and their embryos cultured at the Institute of Molecular Evolution, University of Miami, Coral Gables, Florida. Strongylocentrotus purpuratus and Dendraster excentricus were collected in the vicinity of Friday Harbor Marine Laboratories, University of Washington, Friday Harbor, Washington. Embryos were cultured using the facilities of the Friday Harbor Laboratories. The embryos were cultured near the area of collection in order to obtain material in optimal biological condition. Lyophilized embryonic material was then transported to the University of Florida, Gainesville, Florida, where the rest of the work was carried out.

### Culturing of Embryos

Spawning was induced by the injection of 0.57 M KCl. The eggs were washed two or three times with filtered sea water. Extreme care was taken to avoid the early exposure of the eggs to sperm. Eggs were examined microscopically immediately prior to the addition of sperm to insure that only those egg suspensions in which no visible sperm or fertilization membranes were present were used. The fertilized eggs were

[1]The following symbols will be used in the text to indicate the species of adult echinoid being discussed: L for L. variegatus, T for T. esculentus, S for S. purpuratus, and D for D. excentricus.

washed extensively to free them from excess sperm. This was especially important for the hybrids, since much higher concentrations of sperm were necessary to insure a high percentage of fertilization.

The various combinations of eggs and sperm used to produce embryos for use as antigens, along with the stages of development at the time of harvest, are listed in Table 1. The embryos were maintained in dilute culture with periodic washing. Sea urchins from Florida and Washington were raised at 25°C and 12°C respectively.

## Preparation of Antigens

Embryos of the stages listed in Table 1 were concentrated by centrifugation, washed with sea water, and lyophilized in small aliquots. All lyophilized material was kept at -20°C until used.

Several tissues other than whole embryos were prepared for use as antigens. Jelly-free unfertilized L eggs were prepared by treatment with acid sea water. The eggs were centrifuged from the sea water and lyophilized. Unfertilized eggs of L, T, S, and D with jelly coat intact were also lyophilized. Whole undiluted sperm of L were lyophilized without further processing. L adult gut was dissected from freshly collected animals, washed in several changes of ice-cold sea water, and lyophilized. Coelomic fluid was collected with a syringe, free from sperm or eggs, from L adults and allowed to clot. The clot of phagocytic coelomocytes was centrifuged from the coelomic fluid, and the clot and the fluid lyophilized separately. Fertilizin was prepared from L eggs by washing the freshly spawned eggs once in ice-cold sea water, suspending them in acid

Table 1

Embryonic Stages Used

| Species | | Symbol Used | Hours of | |
|---|---|---|---|---|
| Female | Male | in Text | Development | Embryonic Form |
| L | L | LL | 22 | Prism larva |
| | | | 30 | Pluteus larva |
| T | T | TT | 23 | Mesenchyme blastula |
| | | | 27 | Gastrula |
| | | | 37 | Prism larva |
| L | T | LT | 21 | Early gastrula |
| | | | 25 | Late gastrula |
| | | | 27 | Completed gastrula |
| S | S | SS | 24 | Hatched blastula |
| | | | 42 | Gastrula |
| | | | 70 | Pluteus larva |
| D | D | DD | 18 | Hatched blastula |
| | | | 22 | Mesenchyme blastula |
| | | | 49 | Pluteus larva |
| S | D | SD | 27 | Hatched blastula |
| | | | 47 | "Gastrula" |
| | | | 71 | "Prism larva" |

sea water with a final pH of 4.2 - 4.5, and then centrifuging to remove the eggs. The acid sea water containing the fertilizin was brought to pH 8.0 with 1.0 N NaOH. The fertilizin solution was chilled to 0°C in a salt-ice bath. Five volumes of ethanol at 0°C was added slowly with stirring for each four volumes of fertilizin solution. The resulting white fluffy precipitate was centrifuged from solution, redissolved in a small volume of sea water, and dialyzed in the cold against several changes of sea water. Fertilizin preparations were tested for sperm agglutination titer before use as antigens. All preparations were active in dilutions of at least 1:100.

Am 85 S ribosome fraction was prepared according to the method outlined by Brookbank and Yonge (1964). Unfertilized eggs were treated with acid sea water to remove fertilizin, washed in sea water, and washed in 0.01 M tris-$MgCl_2$-KCl buffer (0.01 M tris [hydroxymethyl] amino methane, 0.01 M $MgCl_2$, 0.2 M KCl), pH 7.9. A 10-20 percent suspension of eggs in buffer at 0°C was expelled repeatedly from a syringe until cell-free. The brei was centrifuged at 11,400 $g_{(max)}$ for 20 minutes in a Spinco Model L2 ultracentrifuge. The supernate was centrifuged again at 11,400 g for 20 minutes. The resulting supernate was centrifuged at 200,000 $g_{(max)}$ for 30 minutes and the ribosomal pellet washed and resuspended in buffer. The 200,000 g centrifugation was repeated twice. Ribosomal preparations were either used fresh or were stored overnight at 4°C.

Preparation of Antisera

Antisera were prepared to 30-hour LL pluteus larvae, 37-hour TT prism larvae, 21-hour LT hybrid early gastrulae,[1] 70-hour SS pluteus larvae, 49-hour DD pluteus larvae, and 71-hour SD hybrid "prism larvae." Lyophilized injection antigens of the various embryos were prepared by suspension in either 0.01 M tris-$MgCl_2$-KCl, pH 7.4 (LL, TT, LT) or 1 percent (w/v) NaCl (SS, DD, SD). An antigen suspension was emulsified in complete Freund's adjuvant (Difco Laboratories, Lot No. 0638) for subscapular injection into New Zealand white male rabbits (previously bled to provide normal serum). Two rabbits were used for each embryonic type except for 21-hour LT hybrid early gastrula for which three rabbits were injected. The SS, DD, and SD series received additional injections in 1 percent (w/v) NaCl (without adjuvant) weekly. Subcutaneous, intramuscular, and intraperitoneal routes were used successively for 3 weeks. Two weeks following the final (intraperitoneal) injection, the rabbits were given an intravenous challenge to increase the antibody titer and bled by cardiac puncture for immune serum 5 days later. The LL, TT, and LT series were given an intravenous challenge 6 weeks after the first adjuvant injection and bled by cardiac puncture at 5 and 12 days after challenge for immune serum.

Normal and immune blood from all rabbits was processed according to the same procedure. The blood was allowed to stand covered at room

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[1] The 21-hour LT hybrid gastrula was the oldest LT hybrid stage available in quantity at the time the antisera were prepared. However, it was expected that if "hybrid" proteins were present, their synthesis would have begun before the gastrula stage.

temperature for 1 hour in test tubes. The clots were separated from the sides of the tubes, and the blood was refrigerated overnight to allow the clot to retract. Serum was obtained by centrifugation and stored frozen until used.

Absorptions of Sera

Control sera and antisera were absorbed with lyophilized whole embryos by dissolving the particulate matter directly in the sera. The sera were refrigerated for 24 hours with frequent mixing, centrifuged to remove the antigen-antibody complexes, and the serum dialyzed in the cold against several changes of 1 percent (w/v) NaCl. Sera were reabsorbed when tests indicated that the first absorption was incomplete.

Absorptions with ribosome preparations were performed by first dialyzing the control and immune sera against 0.01 M tris-$MgCl_2$-KCl buffer, pH 7.9, suspending the ribosomal pellet in the serum to be absorbed, and refrigerating it for 24 hours with frequent mixing. The sera were then centrifuged at 200,000 g for 30 minutes in a Spinco Model L2 Ultracentrifuge in order to remove the precipitate and any excess ribosomal material. The sera were dialyzed in the cold against several changes of sea water for testing on live material or against 1 percent (w/v) NaCl for use in double diffusion plates.

Immune and control sera to be absorbed with whole unfertilized eggs were first dialyzed under refrigeration against several changes of sea water. The unfertilized eggs were treated with acid sea water as in the fertilizin preparation procedure, to remove the jelly, washed twice with

pH 8.0 sea water, and then gently suspended in an equal volume of immune or control sera. The sera were placed in the refrigerator for 1 hour during which the eggs were kept suspended by frequent gentle shaking. The sera were centrifuged to remove the eggs and any precipitate, then used for testing on live material, or dialyzed against 1 percent (w/v) NaCl for use in double diffusion plates. In the absorption procedures with both the ribosomes, and the whole eggs, immune sera were carried through all dialyses, but were not absorbed, in order to provide controls for loss of antibodies through dilution. Control absorption with unfertilized eggs was performed as above on a 0.1 percent (w/v) solution of Rabbit Gamma Globulin, Fraction IV, to check for nonspecific precipitation of globulin by the antigen. Protein determinations were done on absorbed and unabsorbed globulin and sea water controls both before and after absorption.

## Extraction Procedure

Extraction methods utilizing several different salt concentrations and procedures were tested. The following procedure, which gave the most satisfactory results, was used. The lyophilized material was dissolved in 0.17 M NaCl and extracted by vigorous agitation with a vortex mixer. The suspension was allowed to extract in the cold for three hours with periodic vigorous mixing. The suspension was extracted further, in the cold, by repeated expulsion through an 18-gauge needle using a 1-cc syringe. The brei was then centrifuged to remove particulate matter and either used as is or dialyzed against 0.17 M NaCl before use. Protein concentrations

were adjusted to 1-2 mg/ml for most procedures.

In one series of experiments the extraction procedure outlined in Harding, et al. (1954), was duplicated as nearly as possible. Lyophilized material was suspended in 0.14 M NaCl buffered to pH 7.4 with 0.05 M phosphate buffer. The suspension was stirred vigorously for 2 hours and the preparation centrifuged for 1 hour at 25,000 g. The supernate was dialyzed against 0.14 M NaCl for 24 hours. The sediment was washed with 0.14 M NaCl and then extracted with 2 M NaCl buffered to pH 7.4 with phosphate buffer. After extraction with stirring for 2 hours, the suspension was centrifuged as above and the supernate dialyzed against 2 M NaCl for 24 hours. All operations were carried out at 4°C.

## Protein Determinations

Protein determinations were carried out on samples of the extracts according to a procedure modified from Nayyar and Glick (1954). A 4.3 µl sample of a standard solution of bovine serum albumin or protein extract was added to 25.8 µl of bromsulfalein reagent (0.02 percent bromsulfalein in 0.4 N HCl + 0.2 N citric acid), mixed well, and centrifuged at 12,000 g in a Beckman-Spinco 152 Microfuge for five minutes to precipitate the dye-protein complex. A 12.4 µl sample of the supernate was placed in 241 µl of 0.1 N NaOH. Two 100 µl aliquots of each sample were read at 580 mµ in a Beckman-Spinco Microspectrocolorimeter. The optical density readings were converted to mg (bovine serum albumin) protein using a standard curve.

## Preparation of Ouchterlony Plates

Double diffusion plates were prepared using this author's modification, described below, of the Ouchterlony technique (Ouchterlony, 1958). The plates were prepared using 0.3 percent (w/v) Agarose (Gallard Schlesinger Corporation) dissolved in 0.5 percent (w/v) NaCl containing aqueous merthiolate at 1:10,000. The autoclaved agar was poured to a depth of two mm into plastic Petri plates, 55 mm in diameter, to which a thin layer of silicone grease had been applied to prevent seepage between wells. After the agar had set, one center well and six surrounding wells, each 3.6 mm in diameter, were cut equidistant from each other, 8.5 mm from center to center, with the aid of a plastic template and a No. 1 cork borer. The agar was removed from the wells and the plates allowed to synerese for 2-24 hours before removing the fluid and charging the wells with antigens and antisera, approximately 20 µl per well. The plates were maintained at 20°C in a humid chamber. Precipitin bands developed after approximately 12 hours. The plates were observed several times daily and the bands sketched daily until there was no further change in the pattern. The plates were either photographed without staining or washed, dried, and stained with 0.1 percent amidoschwartz in 0.1 M acetate buffer, pH 4.8, and photographed. Some dried, unstained plates were treated with 0.1 percent (w/v) toluidine blue, pH 4.2.

## Immunoelectrophoresis

Microimmunoelectrophoresis was performed essentially as described by Grabar (1959). Barbital buffer, 0.05 M, pH 8.6, was used in the

electrophoresis, to extract the antigens, and to make up the agar. Agarose, one percent, with merthiolate 1:10,000 was poured to a depth of one mm on glass slides 26 x 102 mm. Two antigen wells, 1.5 mm in diameter, were cut 25 mm from the cathode end and 6 mm on either side of the center of the slide. Two microliters of antigen solution containing approximately 30-40 μg of protein were placed into each well and electrophoresis at a constant voltage of 6 v/cm carried out for 90 minutes. After electrophoresis was complete an antiserum well 2 x 90 mm was cut down the center of the slide between the two antigen wells and charged with 0.2 ml of antiserum. The bands were allowed to develop at 20°C in a humid box for 12 hours and the arcs sketched periodically thereafter until no further change was noted.

Passive Cutaneous Anaphylaxis

Passive cutaneous anaphylaxis is an extremely sensitive test for antigen-antibody reactions which was applied as a supplement to the Ouchterlony and electrophoretic examinations.

The fur was clipped from the backs of two albino male guinea pigs and 0.1 ml of various control and immune sera injected intracutaneously into eight numbered sites, four on each side of the backbone. The intracutaneous injections were followed after four hours with an intracardiac injection of 1.0 ml of antigen solution in 1 percent (w/v) NaCl containing about 1.0 mg of protein, and 1.0 ml of 1 percent (w/v) Evans blue in 1 percent (w/v) NaCl. The guinea pigs were scored for positive reactions (a blue spot at the site of antiserum injection) 20 to 60

minutes later. The animals were then killed, skinned, and the under surface of the skin examined to verify scoring.

Tests for Cleavage Inhibition

Various unabsorbed and absorbed control and immune sera were tested for cleavage-blocking activity, using LL embryos. Fertilization membranes were removed from LL eggs two minutes after fertilization by slowly pulling a suspension through an 18-gauge needle fitted to a 50 cc syringe. A 10 µl sample of the washed egg suspension containing about 500 eggs was placed on a glass slide. Ten microliters of the serum or serum dilution to be tested was added. A coverslip was placed over the drop, supported by a 20 mm square rim of vaseline, and sealed to the slide. This operation was completed 20 minutes before the first cleavage. Sera were said to possess cleavage-blocking activity if the first cleavage was completely inhibited in 70 percent or more of the fertilized eggs. Cleavage of fertilized eggs in sea water of in preinjection serum was not inhibited. Cleavage of fertilized eggs in full-strength homologous immune serum was almost 100 percent inhibited. Stepwise dilution produced less inhibition with a sharp drop to well below 70 percent inhibition as the endpoint.

## III. RESULTS

Tables 2 and 3 give comparisons of the times needed to attain various developmental stages for all types of normal and hybrid embryos cultured.

Table 2

Development of LL, TT, and LT Hybrid Embryos at 25°C

| | Total Hours | | | TT | LT |
|---|---|---|---|---|---|
| Stage | LL | TT | LT | LL | LL |
| Two cell | 1.0 | 1.5 | 1.0 | 1.5 | 1.0 |
| Four cell | 1.3 | 1.8 | 1.3 | 1.4 | 1.0 |
| Eight cell | 1.9 | 2.5 | 1.9 | 1.3 | 1.0 |
| Hatched blastula | 10.0 | 11.5 | 10.0 | 1.2 | 1.0 |
| Primary mesenchyme formed | 12.7 | 16.3 | 14.0 | 1.3 | 1.1 |
| Beginning gastrula | 15.7 | 23.7 | 18.9 | 1.5 | 1.2 |
| Prism larva | 22.0 | 37.0 | 37.0 | 1.7 | 1.7 |

Hybrid development in the LT cross is uniform. A long-armed pluteus with a complete gut and skeleton is formed although a longer time is required to reach this stage than is needed in the maternal parent LL. Echinochrome pigment granules, which appear in LL embryos at about 18

hours and in TT embryos at about 20 hours, were not visible in LT hybrid plutei which had developed as long as 90 hours. Although previous descriptions of this hybrid made no reference to the presence or absence of echinochrome, it is evident that production of this pigment is either greatly reduced or lacking. The LT hybrid has double skeletal rods in the anal arms (Fig. 1). This skeletal type is characteristic of the paternal parent, TT, in contrast to the maternal parent which has single anal rods (Figs. 2, 3). In addition, TT has cross connections between the rods which do not occur in the hybrid up to 90 hours of development.

Table 3

Development of SS, DD, and SD Hybrids at 12°C

| Stage | Total Hours | | | DD | SD |
|---|---|---|---|---|---|
| | SS | DD | SD | SS | SS |
| Two cell | 2.4 | 1.6 | 2.4 | 0.7 | 1.0 |
| Hatched blastula | 24.0 | 18.5 | 24.0 | 0.8 | 1.0 |
| Primary mesenchyme formed | 30.0 | 21.0 | 30.0 | 0.7 | 1.0 |
| Beginning gastrula | 36.0 | 24.0 | 40.0* | 0.8 | 1.1 |
| Prism | 46.0 | 36.0 | 59.0° | 0.8 | 1.3 |

*Embryos are viable but exhibit a wide range of variability beginning with this stage.

°The earliest time at which any of the individuals in the population assume this shape. In addition, the culture contains many aberrant forms.

## Legends for Figures

Fig. 1 - LT hybrid pluteus, 54 hours (200x). The arrow points to a double anal rod.

Fig. 2 - TT pluteus, 52 hours (200x). The arrow points to a double anal rod.

Fig. 3 - LL pluteus, 50 hours (200x). The arrow points to a single anal rod.

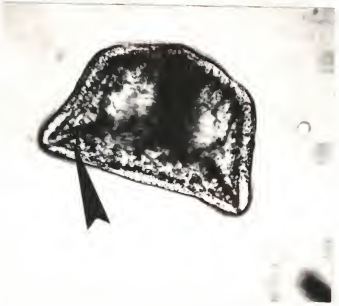

Figure 1

Figure 2

Figure 3

Development of the SD hybrid is maternal and uniform through hatching. Development after primary mesenchyme formation is differentially delayed in individual embryos of the same culture (and age) whether the culture was derived from eggs of a single female or those of several females. For example, the extent of invagination of the archenteron, the shape, the amount of skeletal material, and the amount of pigment varies, apparently independently, among embryos of a given age. The final overall pluteus form which is attained by some of the hybrid population at about 96 hours varies between the maternal and paternal extremes. The remainder of the embryos show intermediate or ovoid shapes not resembling the plutei of either parent (Fig. 4). All the hybrid embryos are vigorous swimmers. Dead or dying embryos are notably absent.

The skeletal rods of the SD hybrid are usually double, and frequently fenestrate (Fig. 4). The condition of the hybrid approximates that of the paternal parent, DD, whose anal skeletal rods are double and fenestrate (Fig. 6). However, in the hybrid the body rod is often double as well as the anal rod. The maternal parent, SS, has only single skeletal rods (Fig. 5).

## Ouchterlony Analyses

Reactions of anti-TT prism larva serum are presented in Table 4. Control preinjection sera were tested in this and all other Ouchterlony analyses and gave no bands with any of the antigens tested. The heterologous reactions with LL and LT embryos produce strong well-defined bands. The homologous reaction of TT embryonic stages with anti-TT pluteus serum

## Legends for Figures

Fig. 4 - SD hybrid pluteus, 96 hours (40x). The arrows point to double rods.

Fig. 5 - SS pluteus, 70 hours (40x). The arrow points to a single anal rod.

Fig. 6 - DD pluteus, 53 hours (40x). The arrow points to a double anal rod.

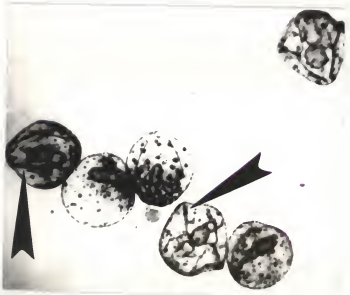

Figure 4

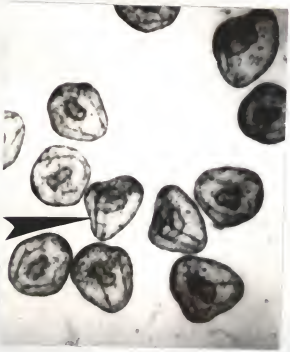

Figure 5

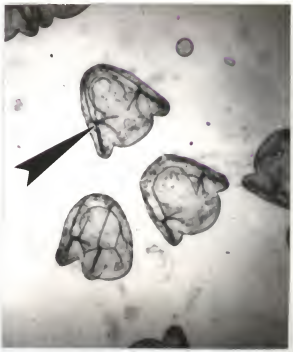

Figure 6

was unchanged by absorption of this serum with L unfertilized eggs. However, the absorbed serum no longer cross-reacted with the embryonic stages of LL or the LT hybrid tested. None of the stages of TT embryos tested, mesenchyme blastula, gastrula, or pluteus, reacted with anti-TT prism larva serum which had been completely absorbed with T unfertilized eggs (Fig. 7).

Table 4

Precipitin Bands Present in Ouchterlony Plates Using Anti-TT Prism Larva Serum

| Antigens | Precipitin Bands Present* | | |
|---|---|---|---|
| T unfertilized egg | a | b | c |
| TT mesenchyme blastula | a | b | c |
| TT gastrula | a | b | c |
| TT prism larva | a | b | c |
| L unfertilized egg | a' | b' | |
| LL prism larva | a' | b' | |
| LL pluteus larva | a' | b' | |
| LT early gastrula | a' | b' | |
| LT gastrula | a' | b' | |
| LT completed gastrula | a' | b' | |

*The letters (a, b, c) are used to indicate relationships to a particular antiserum described within one table. They do not indicate relationships between bands in different tables. Primes are used to designate heterologous antigen-antibody precipitates. The band nearest the antigen well is designated a in all cases, with bands further away given subsequent letters.

Reactions of anti-LL pluteus serum with the various stages of hybrid and homologously fertilized embryos are shown in Table 5 and Fig. 8. The heterologous reactions with TT embryos were strong and produced well-defined bands. Absorption of anti-LL pluteus serum with T unfertilized eggs removed the heterologous reaction of this serum with T unfertilized eggs and TT embryonic stages. LL and LT embryonic stages continued to react with the heterologously absorbed serum producing the same bands; see Table 5. Absorption of the anti-LL pluteus serum with L unfertilized eggs removed all precipitin bands formed with the other embryonic stages of LL and the LT hybrids tested. Increased antigen concentration often resulted in the resolution of a single band complex (i.e. band b, Fig. 8) into two bands, when the plates were observed before the reactions were complete. Such bands fused into one band complex after a longer period of reaction. All stages tested were uniform in this behavior.

Reactions of anti-LT early gastrula serum are presented in Table 5 and Fig. 9. This serum reacted in a manner identical to the anti-LL pluteus serum. Absorption of the serum with L unfertilized eggs removed all reaction with the stages of LL, TT, and LT hybrid embryos tested.

## Legends for Figures

Fig. 7 - Antigen-antibody reactions obtained with antiserum to TT prism larvae (center well). Peripheral wells contain 0.17 M NaCl extracts of L unfertilized eggs (1), T unfertilized eggs (2), TT prism larvae (4), and LT hybrid completed late gastrulae (5).

Fig. 8 - Antigen-antibody reactions obtained with antiserum to LL plutei (center well). Peripheral wells contain 0.17 M NaCl extracts of T unfertilized eggs (1), L unfertilized eggs (2), LL pluteus larvae (4), and LT hybrid completed late gastrulae (5). The arrow points to the b band (Table 4).

Fig. 9 - Antigen-antibody reactions obtained with antiserum to LT early gastrulae (center well). Peripheral wells as in Fig. 8.

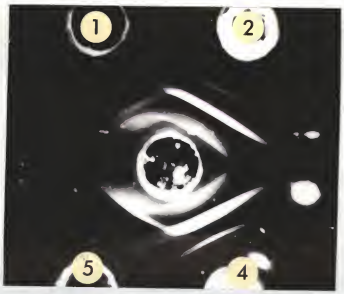


Figure 7

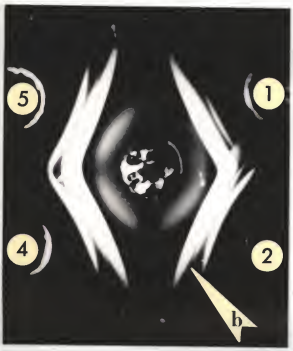


Figure 8

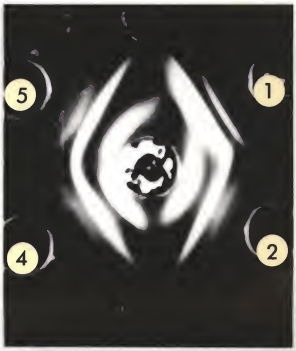


Figure 9

Table 5

Precipitin Bands Present in Ouchterlony Plates, Using Anti-LL Pluteus Serum or Anti-LT Early Gastrula Serum

| Antigens | Precipitin Bands Present* | | |
|---|---|---|---|
| L unfertilized egg | a | b | c |
| LL prism larva | a | b | c |
| LL pluteus larva | a | b | c |
| T unfertilized egg | a' | b' | |
| TT mesenchyme blastula | a' | b' | |
| TT gastrula | a' | b' | |
| TT prism larva | a' | b' | |
| LT early gastrula | a | b | c |
| LT gastrula | a | b | c |
| LT completed gastrula | a | b | c |

*The bands are designated by letters in the manner noted in Table 4.

Reactions of anti-DD pluteus serum are described in Table 6 and shown in Fig. 10. The heterologous reactions with SS and SD embryos were weak. Anti-DD pluteus serum, absorbed with unfertilized eggs, gave no precipitin bands with any stage of DD tested. Absorption of the anti-DD pluteus serum with D unfertilized eggs abolished the reaction of this and older stages of DD embryos with the absorbed serum. Antigens from DD embryos, present in mixtures of equal parts of SS pluteus and DD pluteus extracts, were detectable using this serum.

Table 6

Precipitin Bands Present in Ouchterlony Plates Using Anti-DD Pluteus Serum

| Antigens | Precipitin Bands Present* | | |
|---|---|---|---|
| D unfertilized egg | a | b | c |
| DD hatched blastula | a | b | c |
| DD mesenchyme blastula | a | b | c |
| DD pluteus larva | a | b | c |
| S unfertilized egg | a' | b' | |
| SS pluteus larva | a' | b' | |
| SD hatched blastula | a' | b' | |
| SD "gastrula" | a' | b' | |
| SD "prism larva" | a' | b' | |

*The bands are designated by letters in the manner noted in Table 4.

The reactions of anti-SS pluteus serum with hybrid and homologously fertilized embryos are presented in Table 7 and Fig. 11. This serum gave no visible precipitin bands when tested with DD embryos. Absorption of the anti-SS pluteus serum with unfertilized S eggs abolished all reaction with the S eggs and older stages of SS embryos tested.

Precipitin bands produced by reacting anti-SD "prism larva" serum are presented in Table 8 and Figure 12. The serum gave no heterologous reaction with DD embryos. Antisera were prepared to S unfertilized eggs

Table 7

Precipitin Bands Present in Ouchterlony Plates, Using Anti-SS Pluteus Serum

| Antigens | Precipitin Bands Present* | | |
|---|---|---|---|
| S unfertilized egg | a | b | c |
| SS hatched blastula | a | b | c |
| SS gastrula | a | b | c |
| SS pluteus larva | a | b | c |
| D unfertilized egg | no reaction | | |
| D pluteus larva | no reaction | | |
| SD hatched blastula | a | b | c |
| SD "gastrula" | a | b | c |
| SD "prism larva" | a | b | c |

*The bands are designated by letters in the manner noted in Table 4.

and D unfertilized eggs, but will not be discussed since they gave no additional information.

Various methods of extraction and salt concentrations, including those outlined by Harding, et al. (1954), were tested in Ouchterlony analyses using several of the above antigens and antisera. No additional precipitin bands were found using these alternate procedures.

## Legends for Figures

Fig. 10 - Antigen-antibody reactions obtained with antiserum to DD pluteus larvae (center well). Peripheral wells contain 0.17 M NaCl extracts of S unfertilized eggs (1), D unfertilized eggs (2), DD pluteus larvae (4), and SD "prism" larvae (5).

Fig. 11 - Antigen-antibody reactions obtained with antiserum to SS pluteus larvae (center well). Peripheral wells contain 0.17 M NaCl extracts of D unfertilized eggs (1), S unfertilized eggs (2), SS pluteus larvae (4), and SD "prism" larvae (5). The arrow points to the b band which is double here. This is similar to the condition found with anti-LL pluteus serum (Fig. 8).

Fig. 12 - Antigen-antibody reactions obtained with antiserum to SD "prism" larvae (center well). Peripheral wells as in Fig. 11.

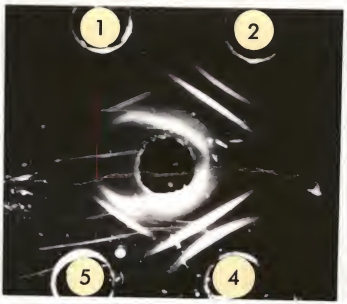


Figure 10

Figure 11

Figure 12

Table 8

Precipitin Bands Present in Ouchterlony Plates, Using Anti-SD "Prism Larva" Serum

| Antigens | Precipitin Bands Present* | | |
|---|---|---|---|
| S unfertilized egg | a | b | c |
| SS pluteus larva | a | b | c |
| SD hatched blastula | a | b | c |
| SD "gastrula" | a | b | c |
| SD "prism larva" | a | b | c |
| DD pluteus larva | no reaction | | |

*The bands are designated by letters in the manner noted in Table 4.

Harding, et al. (1954) employed the absorption method outlined by Björklund (1952). In this method, extracts of the absorbing antigen were allowed to diffuse into the agar for at least 24 hours before the wells were cleaned and filled with antigens and antisera in the normal fashion. Experiments with this technique utilizing anti-SS pluteus serum and extracts of S unfertilized eggs as the absorbant, or anti-DD pluteus serum and extracts of D unfertilized eggs as the absorbant, gave results identical with those obtained with the standard method of particulate absorption. That is, the absorbed sera no longer reacted with the stages of homologous antigen tested.

The reactions of antiserum prepared to LL pluteus with antigens other than whole embryos were determined. These data are presented in

Table 9. Some of the reactions are shown in Fig. 13.

Table 9

Precipitin Bands Present in Ouchterlony Plates Using Absorbed and Unabsorbed Anti-LL Pluteus Serum

| Antigens | Precipitin Bands Present* |
|---|---|
| Unabsorbed Serum | |
| L unfertilized egg | a b c |
| L adult gut | b d |
| L fertilizin | e |
| L adult "blood" clot | no reaction |
| L coelomic fluid ("blood") | no reaction |
| L sperm | f |
| L ribosomes | "a" b c |
| Serum Absorbed With L Ribosomes | |
| L unfertilized egg | a |
| L ribosomes | no reaction |
| Serum Absorbed With Intact Whole Jellyless L Eggs | |
| L unfertilized egg | a b |
| L ribosomes | "a" b |

*The bands are designated by letters in the manner noted in Table 4.

## Immunoelectrophoresis

Microimmunoelectrophoresis was used to confirm the data observed in Ouchterlony plates. Antisera against LL pluteus, TT prism larva, LT gastrula, SS pluteus, and DD pluteus were used. Antigens electrophoresed were embryonic stages of all four urchins and both hybrid crosses. The immunoelectrophoretic patterns produced confirmed the results presented in Tables 4 through 7 with respect to the number of major bands present, the absence of any bands in later embryonic stages other than those found in the unfertilized eggs, and the absence of bands characteristic of the paternal parent or of "hybrid specific" bands in either hybrid embryo.

## Passive Cutaneous Anaphylaxis

One of the two guinea pigs used in this test died after the last intracardiac injection, and before the histamine release reaction had progressed sufficiently to be scored. The results observed in the other guinea pig are presented in Table 10.

## Tests for Cleavage Inhibition

Anti-LL pluteus serum, dialyzed against sea water, blocked cleavage of fertilized demembranated LL eggs. Control embryos developed normally in preinjection serum. Anti-LL pluteus serum, undiluted and at dilutions of 1/2, 1/4, 1/8, and 1/16 prevented the first cleavage in at least 70 percent of the fertilized eggs. Further dilutions continued to inhibit cleavage but at later divisions.

Table 10

Results of a Test for Paternal Antigens in the LT Hybrid by Passive Cutaneous Anaphylaxis in the Guinea Pig

| Site | Serum | Reaction |
|---|---|---|
| R1 | Preinjection serum from the rabbit used to produce anti-LL pluteus serum | - |
| R2 | Preinjection serum from the rabiit used to produce anti-TT prism serum | - |
| R3 | Anti-LL pluteus serum; unabsorbed; diluted 1/3 | + |
| R4 | Anti-LL pluteus serum; absorbed with T unfertilized eggs; diluted 1/3 | + |
| L1 | Anti-TT prism serum; absorbed with L unfertilized eggs | - |
| L2 | Anti-TT prism serum; unabsorbed | + |
| L3 | Anti-TT prism serum; absorbed with L unfertilized eggs; diluted 1/3 | - |
| L4 | Anti-TT prism serum; unabsorbed; diluted 1/3 | + |

Anti-LL pluteus serum absorbed with whole intact jellyless unfertilized L eggs no longer blocked cleavage. However, it still formed some of the typical precipitin bands (a, b) when reacted with L unfertilized egg extracts as can be seen in Table 9 and Fig. 14.

Anti-LL pluteus serum absorbed with ribosomes blocks the first cleavage division of at least 70 percent of the eggs at all dilutions up to 1/8 (inclusive). At further dilutions it continued to block cleavage but at later divisions. This serum also continued to react with L unfertilized eggs producing band a as presented in Table 9.

## Legends for Figures

Fig. 13 - Antigen-antibody reactions obtained with antiserum to LL pluteus larvae (center well). Peripheral wells contain 0.17 M NaCl extracts of L unfertilized eggs (1), L adult gut (2), L coelomocytes (3), L fertilizin (4), 10,000 g sediment of L unfertilized eggs (5), and L coelomic fluid (6).

Fig. 14 - Antigen-antibody reactions obtained with an 0.17 M NaCl extract of L unfertilized eggs (center well). Antisera in peripheral wells. Anti-LL pluteus larvae absorbed with whole, intact jelly-free unfertilized L eggs to remove the cleavage blocking activity of the antiserum (1). Anti-LL pluteus larva serum, unabsorbed (3,5). The arrow points to band c, present in the reactions of the unabsorbed sera, but absent from the reaction of the absorbed serum.

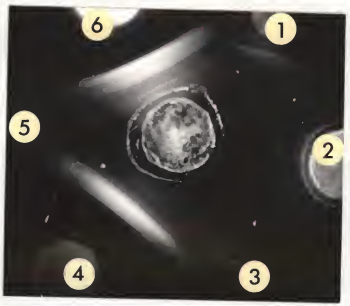


Figure 13

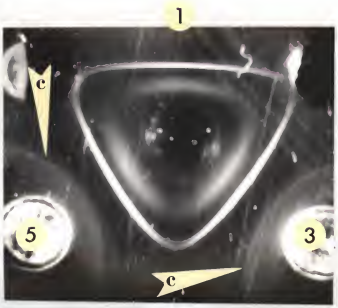


Figure 14

Toluidine Blue Staining

Treatment of washed and dried Ouchterlony plates with 0.1 (w/v) percent toluidine blue, pH 4.2, resulted in metachromatic staining (pink) of certain bands while other bands were either light blue or unstained. Both homologous and heterologous bands of antisera against all four species and the two hybrids were tested. In each homologous reaction the precipitin band closest to the antigen well stained metachromatically, while the other bands stained light blue or were unstained. Heterologous bands stained either metachromatically or not in the same manner as the homologous band to which it was related, that is, to which it was partially fused. Treatment with 0.1 (w/v) percent toluidine blue, pH 2.0, did not stain the bands at all. Specifically, all those bands which had been stained pink at pH 4.2 were now unstained.

## IV. DISCUSSION

The results of this investigation show that in the four species of echinoids studied, no qualitatively new proteins (antigens) are produced, in sufficient quantities to be detected, in the formation of a pluteus larva from a homologously fertilized egg. In addition, the hybrid pluteus larva is formed, in two different crosses, without any evidence that the zygote nucleus and specifically the paternal nucleus, directs the production of any major class or classes of proteins of an antigenic nature. Since most proteins or protein complexes are excellent antigens, this probably means that if the zygote nucleus is directing the production of any qualitatively new proteins in these embryos, they are present in extremely small quantities and do not constitute a major portion of the total protein pool. These results are contradictory to those obtained by Perlmann and his coworkers (Perlmann and Gustafson, 1948; Perlmann, 1953; Harding, et al. 1954). The results obtained on homologously fertilized embryos (Perlmann and Gustafson, 1948; Perlmann, 1953) might be explained on the basis of changes in solubility of proteins already present in the egg, and in any case do not necessarily represent zygote nucleus-directed syntheses. However, the results of Harding, et al. (1954) are definite indications of the activity of the zygote nucleus in directing the production of qualitatively new proteins in quantities sufficient to be detected. Whether their findings can be universally applied to all developing echinoids seems to be in doubt. Thus, mechanisms other than

the synthesis of major amounts of qualitatively new proteins must be considered in determining the processes involved in embryonic differentiation and morphogenesis.

Hybrid Development and Morphology

The first difference from homospermic development displayed by the LT hybrid occurs with the delayed formation of primary mesenchyme. No effects of the sperm nucleus on morphological events in hybrid sea urchin development have been reported and substantiated for stages earlier than the mesenchyme blastula. The timing of the delay in mesenchyme production fits well with the evidence that although m-RNA may be synthesized at the direction of the zygote nucleus as early as the four-cell stage, its activity first becomes necessary to the developing embryo after the blastula hatches.

The developmental delay in formation of the mesenchyme blastula and subsequent stages is not due to lethality typical of hybrids such as P. lividus x A. lixula (Whiteley and Baltzer, 1958), which cease differentiation at the early gastrula stage, since the LT hybrids produce a homogeneous population of well-formed plutei with complete gut and skeleton. Tennent (1911) reports that LT plutei live for at least five days after fertilization. The observations on the development schedule and delay of the hybrid extend those reported by Tennent (1911).

Earlier descriptions of LT hybrid plutei have not dealt with characters other than the skeleton, which is present and normal although showing paternal influences. The only visible indication of abnormality in these plutei is the previously unreported reduction or absence of

echinochrome pigment. This was noted in larvae as old as 90 hours, approximately 70 hours after the first pigment should have been detectable, predicting from observations of either maternal or paternal parent. Young (1958) has studied pigment production in homologously fertilized LL embryos. He reports that gastrulation is necessary to production of pigment, but that the germ layers need not be in their normal relationships. In addition, he reports that the hybrid L. variegatus x A. punctulata or parthenogenetically activated L embryos produce pigment in a pattern and amount not observably different from that of a homologously fertilized LL embryo. Chaffee and Mazia (1953) have reported a limitation by the S. purpuratus sperm of the rate and amount of pigment produced in the hybrid S. franciscanus x S. purpuratus. Griffiths (1965) describes the production of pigments not produced by either parental species in both of the reciprocal hybrids between S. purpuratus and S. droebachiensis. She believes these pigments are either intermediates in the normal pigment synthesis of parental embryos, or pigments produced by a "hybrid enzyme." No biochemical investigations have been made on the echinochrome pigment produced by this hybrid or the paternal species. However, since Young (1958) has shown that the maternal nucleus alone can direct pigment synthesis, the lack of visible echinochrome in LT hybrid plutei may be interpreted to mean that the paternal nucleus interferes with some process normally occurring in the LL embryo leading to pigment production. This might involve the conversion of normal Lytechinus intermediates or echinochromes to colorless compounds related to Tripneustes pigments. Alternatively, the maternal and paternal genomes may produce interacting

products which direct synthesis of a colorless hybrid product not characteristic of either parent.

It is unfortunate that a developmental rate approaching that of the paternal species cannot immediately be interpreted as evidence of the positive action of the paternal genome. One possibility is that the delay represents the increased length of time necessary for an essentially haploid (maternal) nucleus to direct embryogenesis (Loeb, 1899), and that the paternal nucleus is not participating at all. Two observations argue against this possibility. Tennent (1912b) reports no noticeable chromosomal elimination up to the 64-cell stage in counts made on sectioned material from this hybrid. Therefore, the paternal genes are probably present. In addition, the production by the hybrid of double anal skeletal rods similar to those produced by the paternal parent, also reported by Tennent (1911), is a strong indication of action by the paternal genome. Double rods were never seen in control cultures of homologously fertilized LL embryos. A second possible reason for delay may be difficulties of synthesis of Tripneustes end products in the heterologous cytoplasm, or competition between syntheses directed by the two genomes. A third possibility is that the paternal genome may be directing the synthesis of products necessary for embryogenesis at times typical for the nucleus in its homologous cytoplasm. Although it is impossible to decide, on the available evidence, between these last two possibilities, the fact that in the later stages after gastrulation the times for the development of the hybrid closely approach those of the paternal species may be more than merely coincidental.

Development of the SD hybrid is more complex. Primary mesenchyme formation occurs at the same time in the hybrid as in the maternal parent. However, gastrulation is delayed and a morphological variation among individuals in the same culture begins at this stage. Since the paternal species develops faster than the maternal species, delay certainly cannot be interpreted as a positive influence of the sperm nucleus. The development of the hybrid at rates slower than those of either parent is not attributable to lethality, since the SD hybrid has been cultured for at least 12 days after fertilization (Flickinger, 1957), and the embryos gastrulate, form pigment, form skeleton with paternal characteristics, and swim vigorously. Moore (1957) made careful observations on the developmental times and morphology of the SD hybrid. He reported the delay, the wide variation in development, and the paternal skeletal characteristics observed by this author. Flickinger (1957) assayed the alkaline phosphatase activity of the SD hybrid plutei and found it to be intermediate between a high paternal and a low maternal value. He interprets the higher enzymatic activity in the hybrids as evidence of the nuclear control of alkaline phosphatase. No data were given for earlier hybrid stages, so the time of onset of this increased activity is not known. In addition, Flickinger reports that the plutei were maternal in skeleton and morphology on the third day, when the activity assays were made. He has not followed the morphological development of these embryos as closely as other workers.

The DNA content of the SD hybrid hatched mesenchyme blastula is approximately 30 percent greater per unit Kjeldahl nitrogen than that of

the corresponding stage SS embryo. The molecular basis of this elevated level of DNA is currently under investigation (Brookbank, 1968, personal communication). Preliminary results indicate that the increased amount of DNA is not due to a greater amount of DNA per unit Kjeldahl nitrogen for the Dendraster haploid nucleus. One possible interpretation of these data is that asynchronous replication of DNA is occurring with resulting chromosomal imbalance. Such an imbalance could account for the morphological variability observed among these SD hybrid embryos (Boveri, 1902).

Since in both the LT and the SD hybrids, long-lived plutei with paternal characteristics are produced, it is evident that the maternal and paternal genomes can interact successfully within the maternal cytoplasm to produce a considerable amount of development. The SD hybrid, between echinoids from two different orders, shows signs that some difficulties were involved in the production of plutei, since this took longer than in either parent, and since both abnormalities and large variabilities were present. The intermediate developmental time and greater uniformity of the LT hybrid, between echinoderms from two different genera in the same family, may indicate that it had fewer incompatibilities, and needed fewer adjustments. Whiteley (1967) has assessed the binding capacity of newly synthesized RNA from echinoderm larvae, and RNA from unfertilized eggs for single stranded DNA. He tested reciprocal combinations and competition involving RNA from embryos of S. purpuratus, two other urchins of the same genus, the sand dollar, D. excentricus, and a starfish, Pisaster ochraceus. He concludes that the RNA's of unfertilized eggs are much more similar, irrespective of which subclass of

echinoid they are derived from, than is the case with RNA's of prisms of the same species. He concludes that the information needed to control the earlier aspects of embryogenesis, at least to the blastula, is much the same, irrespective of species, and that the larvae-specific messages and the genes that produce them appear to be evolutionarily much more diverse. Thus, with respect to RNA and its products it is to be expected that only in later development would the more severe effects of species differences be manifested, and, from this viewpoint alone, it is predictable that the LT hybrids develop more uniformly and successfully than the SD hybrid.

Many workers have attempted to correlate compatibility between two genomes with the degree of phylogenetic relationship of the parental species of potential hybrids. If this were the only factor involved, the reciprocal crosses should generally exhibit the same degree of developmental success. However, consideration of the reciprocal crosses of these and other species demonstrates that compatibility between the "foreign" genome and the maternal cytoplasm as well as certain physical factors is also involved. The T. esculentus x L. variegatus hybrid forms plutei, but the skeletal characteristics are purely maternal and half the chromosomes (presumably those of Lytechinus) are eliminated by the 16-cell stage (Tennent, 1911, 1912b). No cytological studies have been done on the D. excentricus x S. purpuratus hybrid. However, Flickinger (1957) reports that although the embryos appear normal through gastrulation, they then become abnormal and die. Observations by this author and Brookbank (1968, personal communication) confirm their development through

gastrulation, the beginnings of pigment and spicule formation and subsequent death. Also, Moore (1957) reports that if plutei are formed at all they are maternal. Another pair of sea urchin hybrids demonstrates the disparity of developmental capacity between reciprocal crosses even more clearly. While the combination Sphaerechinus granularis x P. lividus develops into viable plutei with intermediate skeletal characters, without loss of paternal chromosomes, the reciprocal hybrid, P. lividus x S. granularis, dies as a late blastula or early gastrula after elimination of most of the paternal chromosomes in early cleavage (Baltzer and Bernhard, 1955).

Difficulties in cross-fertilization are the primary barrier to the production of hybrids under natural conditions. Given natural or artificial means, by use of increased sperm concentrations or chemical treatment, for achieving any particular cross-fertilization, the success of a particular hybrid is obviously the result of complex interactions between the foreign genome and its surrounding cytoplasm, both in its initial state, and in the changing states produced by the action of the total zygote nucleus. In addition, the activity of the sperm centriole in organizing the mitotic spindle in heterologous cytoplasm; interaction of the paternal chromosomes with the mitotic spindle; energy requirements; chromosomal replication in heterologous cytoplasm, including timing, raw materials, and mechanisms; production of RNA in heterologous cytoplasm; and competition or cooperation between maternal and paternal syntheses must all contribute to the success or failure of embryogenesis by any hybrid.

Parental Antigens

In the current investigation, the antiserum against TT plutei contained antibodies capable of reacting with LL embryos. The two precipitin bands thus formed partially intersect those bands formed between this serum and an antigen of the homologous species. The antibodies produced in response to the TT antigens were reacting with similar antigenic determinants present on molecules from LL embryos having a similar size and shape and thus a similar diffusion coefficient. Heterologous absorption removes the cross-reacting antibodies but does not decrease the number of homologous bands. This suggests that each homologous band represents several different types of antigen-antibody complexes or that each homologous antigenic type stimulates the production of several types of antibodies of varying degrees of specificity or both.

Antigens from LL plutei elicited formation of antibodies capable of reacting with TT embryos. In this case one heterologous band gives a reaction of partial fusion with the homologous precipitin band b, the other heterologous band intersects the homologous band indicating that the antibodies involved are reacting with heterologous antigenic determinants associated with molecules different from those present in the homologous species. Again, heterologous absorption did not decrease the number of homologous bands.

Antisera to DD plutei contained antibodies capable of reacting with SS embryos, although weakly, in a reaction of intersection similar to that seen with one of the heterologous bands produced by the anti LL pluteus

larva antiserum. Antiserum to SS plutei did not cross-react with DD embryos, nor did antiserum to SD "prism" larvae. The fact that cross-reactions between these two species are weak or absent, while those between Lytechinus and Tripneustes are strong, reflects the more distant interordinal relationship between Strongylocentrotus and Dendraster.

The antigens present in the four species of echinoid embryos used in this study were examined qualitatively in order to determine which antigens were characteristic of each species and whether there were losses or gains in antigenic classes accompanying embryogenesis. The precipitin band or band complexes formed by reaction of antipluteus serum with homologous unfertilized eggs or any embryonic stage of the homologous species tested indicate that, in the four echinoids investigated, the detectable antigens or antigen classes present throughout embryogenesis to the pluteus stage do not change either by qualitative loss or gain. This was determined by examination of the reactions of the various embryonic stages with the homologous unabsorbed sera in Ouchterlony and immunoelectrophoretic plates and confirmed by the fact that the antipluteus sera absorbed with homologous unfertilized eggs were no longer capable of reacting with older homologous embryonic stages.

It has been established that transformation of the unfertilized egg to pluteus larva is accompanied by macromolecular syntheses and catabolism. In the present study, the net result of such syntheses, along with any accompanying alterations of antigen solubility, is an antigenic profile which is qualitatively the same throughout the developmental period under investigation, and within the limits of the detection

methods used. Proceeding in time from the unfertilized egg, any or all of the following phenomena might be expected to occur: synthesis of qualitatively new antigens; total degradation of existing antigens; changes in solubility or extractibility; transformations of existing substances to ones of new antigenic specificities or to ones lacking antigenicity. If any of these processes occur, in the developing embryos studied, they must involve a minor portion of the antigenic molecules present within the egg, and are not resolvable by the methods used. The major transformations which do take place during development must either involve nonantigenic molecules, or involve transformations between existing antigenic populations.

Staining of the precipitin bands with toluidine blue was used to characterize, cytochemically, antigens contributing to these precipitates. Preliminary tests using extract or antiserum spots on paper showed that while the extract produced some metachromacy, the antiserum itself did not stain metachromatically. This observation confirms those of other workers that serum globulin does not stain metachromatically with toluidine blue (Baker, 1958), and thus indicates that any metachromacy is due to the reaction of the dye with the antigen itself. Acid mucopolysaccharides are strongly metachromatic, and nucleic acids weakly so (Baker, 1958). However, acid mucopolysaccharides stain metachromatically at pH 2 as well as at pH 4, while ribonucleic acids stain metachromatically only at the higher pH (Cowden, 1968; personal communication). Since those bands which are pink at pH 4 (that is, those closest to the antigen well, such as band a, Table 5) do not stain metachromatically at pH 2, the

metachromacy of these bands is probably due to the presence of ribonucleic acid or ribonucleoprotein antigens, possibly ribosomes or ribosomal subunits, in these bands.

## Hybrid Antigens

It was hoped that with knowledge of the antigens present in each species, investigation of the antigens in the two hybrids might allow the detection of the following possible classes of antigens:

1. Antigens characteristic of the maternal species but not detectable in extracts of the unfertilized maternal egg. These molecules could originate as either newly solubilized molecules previously present in the unfertilized egg in an insoluble form and changed in the course of embryogenesis; molecules synthesized after fertilization on RNA templates present in the unfertilized egg; or molecules synthesized after fertilization from RNA templates produced after fertilization.

2. Antigens characteristic of the paternal species and of those classes present in the unfertilized eggs of the paternal species. Detection of antigens of this type would indicate that new templates are formed during embryogenesis which are identical to those produced during oogenesis.

3. Antigens characteristic of the paternal species and not detectable in the extracts of unfertilized eggs of that species. Suitable absorptions could be used to demonstrate that these antigens were of a qualitatively new type.

4. Antigens characteristic of neither parental species and peculiar to the hybrid (Irwin, 1947). A possible example of this type would be the

formation of a molecule with a new antigenic specificity from subunits synthesized individually by the maternal and paternal genomes, or the alteration of a molecule (structural) synthesized at the direction of one genome by a molecule (enzymatic) synthesized at the direction of the other genome.

It was anticipated that the antigens initially present in the maternal cytoplasm could be detected in the hybrid, and any changes in the normal behavior of the maternal antigen pattern, brought about by the paternal genome, could be detected.

The uniformity of antigenic pattern observed for the different stages of each echinoid studied was observed for the stages of both hybrids when tested with antihybrid gastrula or antihybrid pluteus serum or with antiserum against plutei of the maternal species. This means that hybrid development progresses without production of any further maternal antigenic types, without production of hybrid specific antigens, and without qualitative alteration of the normal pattern of maternal antigens, at levels detectable by these methods. In addition, the lack of reaction of hybrid embryos with antipaternal pluteus sera, heterologously absorbed where necessary to remove cross-reactions with maternal antigens, indicates that although the paternal nucleus appears to participate in the development of both hybrids, antigens characteristic of the paternal species are either not synthesized or are synthesized in amounts too minute to be detectable in precipitin reactions. This result was confirmed for the LT hybrid by passive cutaneous anaphalaxis in the guinea pig, an assay capable of detecting 0.5 μg of total antigen (Ovary, 1958). The anti-TT pluteus

serum used, after absorption to remove cross-reactions with maternal antigens, gave a negative reaction when tested with hybrid late gastrulae. This serum is known to contain antibodies present after heterologous absorption, capable of reacting with those antigens present in Tripneustes throughout development, and thus the negative result means that appreciable synthesis of extractable paternal antigens is not taking place.

The results reported above differ in several important points from those found by Perlmann and his coworkers (Perlmann and Gustafson, 1948); Harding, et al., 1954; Westin, et al., 1967). They found the appearance of qualitatively new antigens in the P. lividus mesenchyme blastulae, and paternal antigens present in later stages of three echinoid hybrids. This difference can be ascribed either to methodology or to differences among the echinoids involved. The author and Perlmann have each used several different schedules of immunization and, as titers have not been given in either case, any direct comparison between the two is impossible. In view of the differing results, an effort was made to duplicate as nearly as possible the extraction procedures, antigen concentrations, and absorption methods reported by Perlmann and his coworkers. Such efforts only confirmed the results reported by this author. In view of the fact that four different species of echinoid have been investigated immunologically in the present study, the possibility certainly exists that in these species, formation of the pluteus is accomplished without the formation of molecules of new antigenic specificity.

## Adult Antigens

The presence in the embryo of antigens similar or identical to those found in adult tissues has been demonstrated by other workers using both antisera to adult tissues (Schechtman, 1948), and antisera to embryonic tissues (Cooper, 1948). The antiserum prepared to LL plutei reacted with adult gut as well as with sperm, while failing to react with either adult coelomocytes or adult coelomic fluid. The antigenic determinants present in the adult gut are apparently related to those participating in one precipitin band complex present when anti-LL pluteus serum is reacted with LL embryo homogenate. These determinants are also related to those present in the ribosomal preparations, since absorption of the anti-LL pluteus serum with a ribosomal preparation removes those antibodies which had previously reacted with adult gut. In this context it is interesting to note that Whiteley, McCarthy, and Whiteley (1966) found that RNA from adult gut and testis compstes with m-RNA from prism larvae in forming complexes with complementary single-stranded DNA. There is evidently a core of messages which is present during oogenesis, embryogenesis, and adult life, which is probably involved in directing those cellular functions common to all cells. It may be that the similar proteins detected here represent the protein products of such RNA synthesis.

## Cleavage Inhibition

One property of antiserum made against various embryonic sea urchin components, most notably fertilizin, is its ability to block the cleavage

of fertilized, demembranated eggs of the homologous species. This property is not found in antisera against whole sperm, or adult tissues such as epidermis, or blood (coelomocytes) (Tyler and Brookbank, 1956). In the current work the antiserum prepared against LL plutei reacts with LL fertilizin. This antiserum also blocks the cleavage of fertilized eggs of this species. Upon absorption with whole intact jellyless unfertilized eggs, cleavage-blocking activity was removed, along with at least one group of antibodies capable of forming a precipitate with LL embryo homogenates. Thus at least one of the precipitin bands (c) is formed by antibodies which can be specifically absorbed by surface components of the unfertilized egg.

It had been previously reported (Brookbank and Yonge, 1964) that the 85 S ribosomal fraction, isolated from unfertilized Lytechinus eggs, was capable of completely absorbing the cleavage-blocking activity from antisera against Lytechinus fertilizin. An attempt was made to absorb the cleavage-blocking activity from the anti-LL pluteus serum, using an 85 S ribosomal fraction of Lytechinus unfertilized eggs prepared as in Brookbank and Yonge (1964) but with additional low-and high-speed centrifugations to remove possible impurities. The antiserum absorbed with this fraction no longer contained antibodies capable of reacting with the ribosomal preparation, but still contained antibodies (at a lower titer than found in the original antiserum) capable of blocking cleavage. It is possible that the antiserum against Lytechinus plutei contains cleavage blocking antibodies of at least two different specificities; one type directed against fertilizin or fertilizin-like haptens (Brookbank, 1959)

and absorbable with the ribosomal preparations, and the other type directed against some other component(s) localized in the egg surface. The cleavage-blocking serum against fertilizin, used by Brookbank and Yonge (1964) would, of course, contain antibodies against only the surface fertilizin component, and might be completely absorbable by the ribosomal preparation. An alternate explanation for the reported difference is that the cleavage-blocking antibodies were absorbed from the anti-Lytechinus fertilizin serum by high molecular weight impurities present in the ribosomal preparations. The purer preparations used to absorb anti-LL pluteus serum did not contain these impurities, and thus did not absorb the cleavage-blocking antibodies.

Metz and Thompson (1967), in testing the effects of antisera against eggs or against fertilizin, made univalent by papain digestion, upon the cleavage of homologous fertilized eggs, report that the titer of cleavage-blocking activity was diminished but not abolished by rendering the antibody univalent. They also suggest the possibility that there are two different egg antigens involved in cleavage inhibition: one which must be involved in a cross-linkage with multivalent antibody to be effective, and the other which can inhibit cleavage by blocking the antigenic sites, without cross-linkage. This assumes that the antibody used was univalent, which seems highly probable from controls, and other tests of the same sera.

Of the three major precipitin bands present in the reaction of unfertilized eggs or embryos with anti-LL pluteus serum, the first or a band appears in reactions with ribosomes. Some of the antibodies which

contribute to this band remain after absorption of the serum with ribosomes, and can continue to react with embryo homogenates. It is postulated that some of the antibodies comprising this band are partially responsible for the cleavage-blocking activity of the antiserum, especially that not absorbable by the ribosomes which may represent the second, nonfertilizin-like component. The second band complex, b, also appears when reacted with ribosomal preparations, and with preparations of adult gut. It is postulated that the antigens forming this band may be involved in some of the more universal functions of cells. The third band complex, c, which disappears completely when cleavage-blocking activity of the serum is totally absorbed with the surface components of unfertilized eggs, probably represents the second cleavage-blocking component, and is comprised of antigens associated with the surface.

The necessity of protein synthesis for development and differentiation in sea urchins and other embryonic systems has been discussed previously. The distribution of those proteins synthesized at a particular period in development changes with respect to the overall protein pattern of the egg, particularly after the blastula hatches (Gross, 1967). Evidently there must be synthesis of new RNA by the zygote nucleus for protein synthesis and development to continue past this point. A study of Xenopus laevis embryos (Denis, 1964) indicates that types of m-RNA are present in late larval embryos which are not detected in earlier developmental stages. Evidence is accumulating that some classes of m-RNA synthesized by sea urchins during oogenesis are synthesized by embryos even at the prism larva stage. These types of m-RNA are also present, to a

limited extent, in adult tissues such as gut and testis (Whiteley et al., 1966). In addition, there are some types of m-RNA present in the gastrula and pluteus (Glisin, Glisin, and Doty, 1966) which are not represented either at earlier stages or in the adult.

There is increasing evidence, both from the present study and from other workers (Spiegel, 1960; Shore, 1965; Spiegel, et al., 1965; Terman and Gross, 1965; Westin, et al., 1967), that qualitatively new proteins, especially those whose production is directed by the zygote nucleus and which may be either involved causally in or result from cell differentiation, occupy quantitatively a small proportion of the total protein population.

It may be postulated that cell differentiation involves not only the synthetic and degradative processes demonstrated by many of the above workers, but also simultaneous rearrangements of the components of the existing protein species, as the result of localized differences in the intracellular environment. These rearrangements would not require new macromolecular syntheses and might involve small molecular weight compounds. Koshland and Kirtley (1966) have reviewed the evidence for the controlled molecular flexibility of enzymes in forming enzyme-substrate complexes, in the process of activation, and with respect to feedback inhibition. Such considerations have led these authors to suggest that stable changes in the conformation of existing proteins may have important effects upon growth and differentiation. These changes in the protein conformation need not alter the antigenic determinants present on such a protein since, while tertiary structure is undeniably important in

antigenic activity, it is also true that denatured, partially denatured, and fragmented proteins still retain some of their antigenic activity. Since any single antigenic determinant probably involves only a few amino acid residues or monosaccharide units (Kabat, 1966), such areas would not necessarily be altered or hidden by such structural rearrangement. Therefore, if differentiation involves appreciable alterations of this kind, such processes would not necessarily be detected as antigenic differences. Thus, small differences in intracellular environment such as pH, ionic strength, cell metabolites, small molecular weight compounds, or elements may produce molecules of altered structure and properties with consequent cell differentiation.

## V. SUMMARY

1. Embryos of the intergeneric hybrid of Lytechinus variegatus eggs and Tripneustes esculentus sperm and the interordinal hybrid of Strongylocentrotus purpuratus eggs and Dendraster excentricus sperm were cultured, their developmental rate and morphology described, and the morphological influence of the paternal nucleus observed.

2. The antigens present in the unfertilized eggs, mesenchyme blastulae, gastrulae, and larvae of the two hybrids and the four parental species were investigated using the Ouchterlony technique and immuno-electrophoresis in conjunction with specific absorption of the antisera.

3. No paternal antigens or "hybrid" specific antigens were detected in extracts of the embryos of either hybrid. Thus, hybrid pluteus larvae of two different crosses are formed without any indication that the zygote nucleus directs the synthesis of any major class of antigenic protein.

4. No molecules of new antigenic specificity were detected in developmental stages of the four parental species investigated.

5. The nature of the antigenic proteins in embryos of one of the parental species, L. variegatus, was characterized more extensively. They include a ribonucleoprotein antigen associated with 85 S ribosomes. In addition, two different antigen types were detected which elicited the formation of antibodies capable of blocking the cleavage of fertilized

sea urchin embryos of the homologous species.

6. Mechanisms of embryonic differentiation were discussed in the light of these results.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Arts and Sciences and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1968

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